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Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces

(pattern formation/morphogenesis/liver CAM/N-cadherin/adherens junction-specific CAM)

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ABSTRACT **Cell adhesion molecules (CAMs) are cell surface glycoproteins that may play a variety of roles in morphogenesis and histogenesis, particularly in defining borders of discrete cell populations. To examine the influence of CAM expression on such cell segregation events *in vitro*, we have transfected cells with cDNAs coding for two calcium-dependent CAMs of different specificity, the liver CAM (L-CAM) and the structurally related molecule N-cadherin. The cDNAs were introduced separately or together into murine sarcoma S180 cells, which normally do not express these molecules, to produce cell lines denoted S180L, S180cadN, and S180L/cadN, respectively. A number of cell lines of each type were produced that differed in their levels of CAM expression. In adhesion assays, S180L and S180cadN cells aggregated specifically via their respective CAMs, and S180L cells did not appear to adhere to S180cadN cells. Cells expressing high levels of each CAM aggregated more rapidly than cells expressing low levels. Segregation between two cell types occurred when they expressed CAMs of different specificity or different levels of the same CAM. S180L and S180cadN cells both sorted out from untransfected cells, and cells expressing high levels of either L-CAM or N-cadherin segregated from cells expressing low levels of the same CAM; in all cases segregation was inhibited by antibodies specific for the transfected CAM. S180L cells sorted out from S180cadN cells, but this segregation was inhibited only when antibodies to both CAMs were applied together. Doubly transfected S180L/cadN cells also sorted out from S180L cells and from S180cadN cells, and the process was inhibited by antibodies to the unshared CAM (N-cadherin or L-CAM, respectively). Cytochalasin D and nocodazole inhibited sorting-out, consistent with the probable role of microfilaments and microtubules in cell movement and in accord with evidence that the action of these CAMs depends on interactions with cortical cytoplasmic components. Using cDNAs for only two CAMs in these studies, we could distinguish at least eight cell lines by their behavior in sorting-out assays. This suggests that qualitative and quantitative differences in the expression *in vivo* of a relatively small number of CAMs can lead to a large variety of patterns among cell collectives and their borders during tissue formation.**

Recent studies on the function and expression of cell adhesion molecules (CAMs) during development have emphasized the significance of these molecules in morphogenesis (for review, see refs. 1 and 2). A critical role for adhesion in development was suggested in past studies by a variety of cellular studies, including the observation that cells of different origins, when mixed in culture, could sort out and produce aggregates that differentiated in accord with the tissue from which they were derived (3–6). Through such sorting-out experiments, attempts were made quantitatively to correlate histological characteristics with the ability of cells derived from given tissues to surround or be surrounded *in vitro* by cells from other tissues (7, 8). Because so little was known about the molecular interactions involved in cell adhesion, however, the models proposed were abstract, and it was not possible to test them in a conclusive fashion.

Over the past decade, a variety of CAMs and their cDNAs have been characterized (2), opening new possibilities for exploring CAM functions and a more fundamental analysis of sorting-out phenomena. A particularly valuable approach has been to transfect CAM cDNAs into cells that do not normally express these molecules. Mouse L cells transfected with cDNAs for neural CAM (N-CAM) and liver CAM (L-CAM; ref. 9) and N-cadherin (10) or P-cadherin (11) express the appropriate CAM and aggregate specifically via that CAM. In addition to inducing cellular aggregation, a transfected CAM can induce cellular shape changes and expression of junctions that are indicative of its morphoregulatory role *in vivo* (12). Moreover, cells transfected with E-cadherin segregated from those transfected with P-cadherin, but cells in these experiments (11) were agglutinated by lectins rather than by CAM bonds alone.

To analyze the qualitative and quantitative role of CAMs themselves in cell sorting, we have produced mouse S180 cell lines that express chicken L-CAM, N-cadherin [also called adherens junction-specific CAM (A-CAM); ref. 13], or both and showed that cells transfected with L-CAM (S180L cells) bind to each other but not to cells transfected with N-cadherin (S180cadN cells) (F. Matsuzaki, R.-M.M., and G.M.E., unpublished data). S180 cells were also transfected with cDNAs for both L-CAM and N-cadherin (S180L/cadN cells). These double transfectants bound to each other via both CAMs and to single transfectants by the shared CAM. In all cases, L-CAM and N-cadherin acted independently. We have expanded this initial set of cell lines in the present study by producing lines that express L-CAM, N-cadherin, and also both of these CAMs at high and low levels and have examined each in specific sorting-out assays. In accord with previous hypotheses (14), we found that segregation between two cell lines can occur when they differ either in the level of expression of the same CAM or in the specificities of CAMs expressed at the cell surface. From the various combinations involving only two CAMs, at least eight populations of cells capable of a distinct sorting-out behavior could be distinguished.

MATERIALS AND METHODS

Reagents. Polyclonal anti-L-CAM IgG and Fab' fragments were prepared (11). Monoclonal anti A-CAM antibody FA-5 (BioMakor, Rehovot, Israel) was used to detect N-cadherin; A-CAM is similar or identical to N-cadherin (15) and therefore we refer to the antibody here as either anti-A-CAM or

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Abbreviations: CAM, cell adhesion molecule; L-CAM, liver CAM; A-CAM, adherens junction-specific CAM.

anti-N-cadherin. Other reagents included polylysine, cytochalasin D, and nocodazole (Sigma), dil and diO (Molecular Probes), human fibronectin (New York Blood Center, New York), and tissue culture media (GIBCO).

Transfections. S180 mouse sarcoma cells (American Type Culture Collection) were transfected with chicken L-CAM and N-cadherin cDNAs to produce cell lines that expressed different levels of these CAMs. Previously designated S180L, S180cadN, and S180L/cadN (ref. 12; F. Matsuzaki, R.-M.M., and G.M.E., unpublished data) are designated L_H, $cadN_H$, and L_HcadN_H in the present study to distinguish their level of expression from that of other lines with lesser levels of expression.

Expression of Transfected CAMs. Aliquots of boiling sodium dodecyl sulfate (SDS) extracts from subconfluent cultures containing 50 μg of protein were loaded per lane in SDS/PAGE. L-CAM was detected on immunoblots with polyclonal antibodies to L-CAM and ^{125}I-labeled protein A; for N-cadherin, monoclonal antibody FA-5, and ^{125}I-labeled sheep anti-mouse IgG were used. The relative amounts of ^{125}I in the L-CAM and N-cadherin bands were determined (16), defining the amount of L-CAM in L_H cells and N-cadherin in $cadN_H$ cells as 1.0. The values for the various lines were: L_L, 0.40; $cadN_L$, 0.24; L_HcadN_H, 0.81 (L-CAM), 0.74 (N-cadherin); L_HcadN_L, 0.92, 0.11; L_LcadN_H, 0.40, 0.76; S180, 0.0, 0.0. Immunofluorescent staining and immunoprecipitation of cell surface proteins labeled by lactoperoxidase iodination confirmed that the CAMs were expressed at the cell surface.

Cell Adhesion Assays. In aggregation assays (17), cells (2×10^5 in 600 μl of Eagle's minimal essential medium without bicarbonate/20 mM Hepes) were shaken in 24-well plates coated with 1% agarose. Cells were obtained from subconfluent monolayers by treating them with phosphate-buffered salt solution/5 mM EDTA/2% fetal bovine serum. Heterotypic binding was determined by counting the number of probe cells in suspension that bound to cells in a confluent monolayer. Twenty-six-well slides (Cel-line Associates, Newfield, NJ) were coated sequentially with poly(L-lysine) (10 μg/ml in distilled water for 30 min) and fibronectin (20 μg/ml for 30 min). Monolayers were formed by incubating 10^4 cells in 10 μl of growth medium (12) overnight. Probe cells were labeled with the fluorescent dye dil (6 μg/ml in medium) for 12 hr, removed from dishes as described above, preincubated in medium with antibodies for 30 min on ice, and then added (10^3 cells in 10 μl) to each monolayer. After 40 min, slides were washed with Hanks' balanced salt solution, fixed in 3.5% formaldehyde, washed in distilled water, and air-dried; labeled cells attached only to cells in the monolayers and not to the few empty spaces in the monolayers. Fluorescence was quantitated with a photometer (Zeiss).

Sorting-Out Assays. Cells were differentially labeled with either dil (3 μg/ml) or diO (10 μg/ml) for 12 hr and were removed from dishes with 0.25% trypsin/2 mM EDTA. Cells (10^3–10^5) in 100–200 μl of medium were added to each V-shaped well of 96-well plates (Dynatech) and blocked with 10 mg of bovine serum albumin per ml, and the cells were pelleted by centrifugation at 250 × *g* for 1 min (18). By monitoring the cultures at different times, it was possible to follow the progress of cell segregation events. Results were recorded as pairs of black and white micrographs obtained with filters appropriate for rhodamine (for dil) and fluorescein (for diO). Examples were presented in pseudocolor obtained by recombining black and white images onto color film with red and green filters.

RESULTS

Characterization of Transfected Lines. cDNAs encoding chicken L-CAM and N-cadherin were transfected separately or in combination into the parent mouse sarcoma cell line S180, and lines expressing different amounts of each CAM were selected. Based on the type and amounts of CAM expressed on their cell surfaces, we were able to distinguish eight different cell lines. Cell lines were designated on the basis of the transfected CAM cDNA (i.e., L, cadN, and L/cadN) with subscripts H and L indicating high and low levels of CAM expression.

Table 1. Aggregation of transfected cells

Cell line	Antibodies* NI	Anti-L-CAM	Anti-N-cadherin	Anti-L-CAM + N-cadherin
S180	21 ± 2	21 ± 1		
L_H	73 ± 2	20 ± 4 (73)		
L_L	39 ± 2	23 ± 3 (42)		
$cadN_H$	46 ± 3		22 ± 2 (53)	
$cadN_L$	30 ± 1		22 ± 1 (27)	
L_HcadN_H	95 ± 1	87 ± 3 (7)	88 ± 0 (7)	61 ± 1 (36)

NI, nonimmune.
*Fab' fragments of nonimmune and anti-L-CAM IgG were used at 250 μg/ml; monoclonal antibody FA-5 was used at a 1:100 dilution. Numbers are averages ± mean deviations ($n = 2$) and represent the % of aggregation; numbers in parentheses are % inhibition of aggregation.

Untransfected S180 cells aggregated weakly in suspension (Table 1). Consistent with the observation (ref. 12; F. Matsuzaki, R.-M.M. and G.M.E., unpublished data) that S180 cells do not express L-CAM or N-cadherin, this "background" aggregation, while calcium-dependent, was not inhibited by antibodies to these CAMs. In contrast, transfected cells exhibited homotypic aggregation (i.e., aggregation of like cells) that was inhibited by Fab' fragments of antibodies specific for each CAM (Table 1). Higher degrees of aggregation were observed with higher levels of expression: L_H cells aggregated more than L_L cells, and $cadN_H$ cells aggregated more than $cadN_L$ cells. Aggregation of cells expressing both L-CAM and N-cadherin was weakly inhibited by either Fab' fragments of antibodies to L-CAM or by monoclonal antibodies to A-CAM (N-cadherin), and maximal inhibition was obtained when both reagents were used, suggesting that both molecules contributed to the aggregation.

To examine adhesion between cells of different transfected lines (i.e., heterotypic adhesion), dissociated cells of one line were added to confluent monolayers of another cell line (Table 2). Both L_H and $cadN_H$ cells bound weakly to untransfected S180 monolayers, but this "background" adhesion was not inhibited by Fab' fragments or monoclonal antibodies to L-CAM and N-cadherin, respectively, consistent with the conclusion that S180 cells do not express a receptor for either L-CAM or N-cadherin. As in the suspension assays, L_H probe cells bound to L_H cell monolayers, and $cadN_H$ cells bound to $cadN_H$ monolayers; the binding was inhibited by Fab' fragments of antibodies to L-CAM and

Table 2. Adhesion of cells to monolayers

Cells in suspension	Cells in monolayer	Antibodies* NI	Anti-L-CAM	Anti-N-cadherin
L_H	S180	22 ± 7	20 ± 3	
	L_H	89 ± 13	21 ± 2	
	$cadN_H$	24 ± 4	24 ± 2	
$cadN_H$	S180	28 ± 5		32 ± 4
	L_H	30 ± 6		23 ± 3
	$cadN_H$	72 ± 15		44 ± 10

NI, nonimmune.
*Specific antibodies were used as described in Table 1. Numbers are averages ± SEM ($n = 3$) and represent cells attached per 1 mm^2.

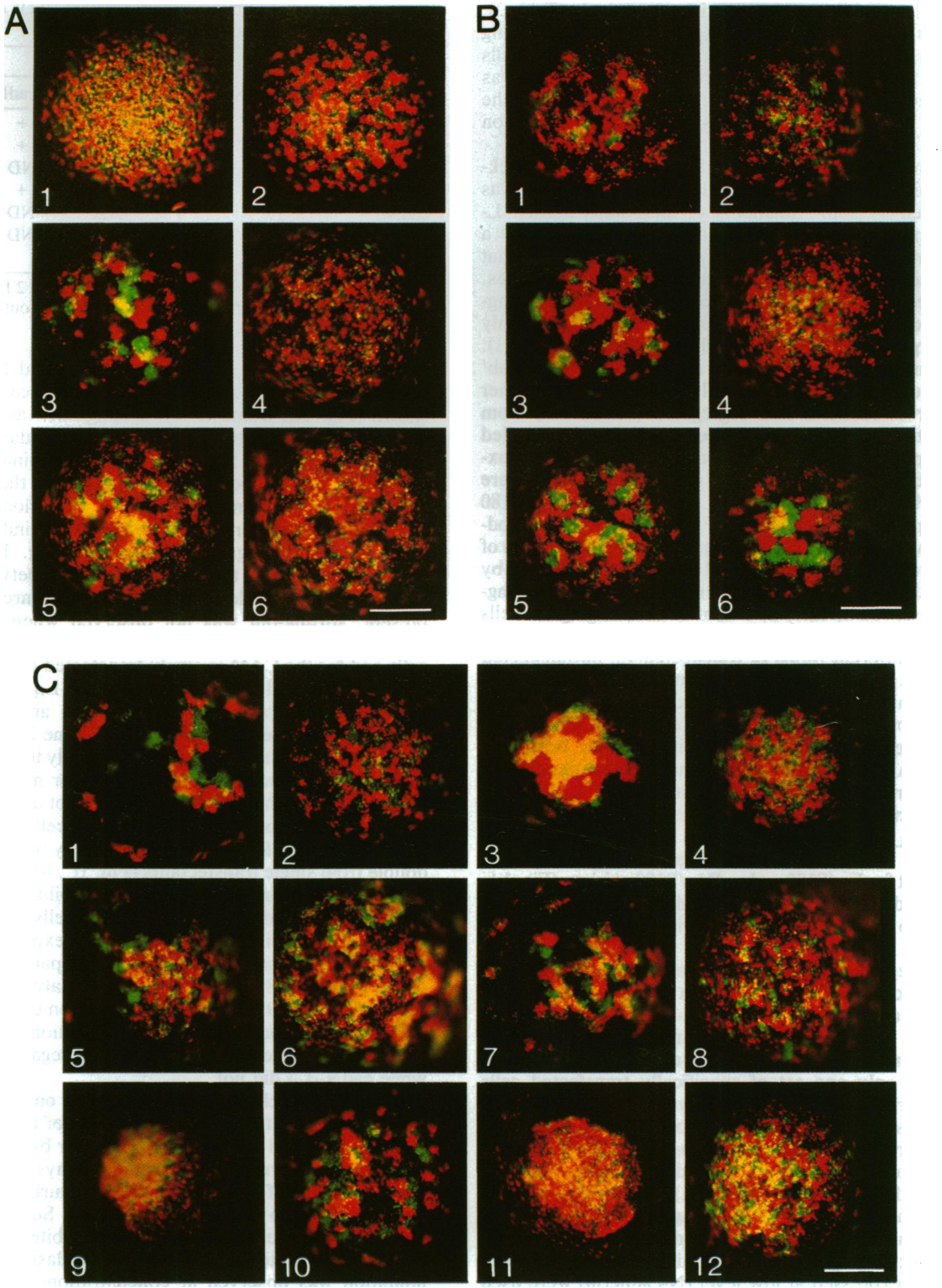

FIG. 1. Micrographs of living cells in sorting-out assays. Cells appear red or green as indicated. Yellow reflects the superposition of red and green of unsegregated cells or a superposition of cells that have segregated but are at different depths; the latter situation was frequently observed at the center of the well where the culture is deepest, producing the large yellow patches in the center of red and green cells that are clearly separated into discrete groups (e.g., see *C*, frame 3). Where indicated, 250 μg of Fab' fragments of nonimmune IgG or anti-L-CAM IgG per ml or a 1:100 dilution of monoclonal antibodies to N-cadherin were used. (Bar = 400 μm). (*A*) Cells expressing a single CAM type. Frames: 1–4, L_H (red) and S180 (green); 5 and 6, $cadN_L$ (red) and S180 (green). Cultures were examined immediately (frame 1) and after a 6-hr (frame 2) and a 16-hr (frames 3–6) incubation with nonimmune (frames 1–3 and 5), anti-L-CAM (frame 4), or anti-N-cadherin (frame 6) antibodies. (*B*) Cells expressing different levels or type of CAMs. Frames: 1 and 2, $cadN_H$ (red) and $cadN_L$ (green); 3–6, L_H (red) and $cadN_H$ (green). Cultures were incubated 16 hr with nonimmune (frames 1 and 3), anti-L-CAM (frame 5), anti-N-cadherin (frames 2 and 6), or a combination of anti-L-CAM and anti-N-cadherin antibodies (frame 4). (*C*) Cells transfected with both L-CAM and N-cadherin. Frames: 1–4, L_HcadN_L (red) and S180 (green); 5–8, L_HcadN_L (red) and $cadN_L$ (green); 9–12, L_HcadN_H (red) and L_H (green). Cultures were incubated 16 hr with nonimmune (frames 1, 5, and 9) anti-L-CAM (frames 2, 6, and 10), anti-N-cadherin (frames 3, 7, and 11), or a combination of anti-L-CAM and anti-N-cadherin antibodies (frames 4, 8, and 12).

monoclonal antibodies to N-cadherin, respectively (Table 2). In contrast to the results of suspension assays, binding between L_L cells and L_L monolayers or between $cadN_L$ cells and $cadN_L$ monolayers was at the background level and was not inhibited by specific antibodies, suggesting that while the monolayer assay is qualitatively similar to the suspension assay, it is less sensitive.

Adhesion between L_H and $cadN_H$ cells was at the background level seen for untransfected cells. This adhesion was not inhibited by Fab' fragments of antibodies against L-CAM, monoclonal antibodies against N-cadherin, or by a combination of both, consistent with the conclusion that L-CAM and N-cadherin have different binding specificities.

Cell Segregation. When observed at the start of a sorting-out assay, cells at the bottom of wells were mixed uniformly without large clusters of either cell type (Fig. 1*A*, frame 1). After 16 hr in medium alone or in the presence of Fab' fragments of nonimmune IgG, cells transfected with either L-CAM or N-cadherin had segregated (sorted out) from untransfected cells (Fig. 1*A*, frames 3 and 5). The detailed geometric pattern differed in replicate wells, but the approximate number of large clusters and overall morphology were reproducible. The segregation of L_H cells from parent S180 cells was specifically inhibited by Fab' fragments of antibodies to L-CAM (Fig. 1*A*, frame 4); similarly, the segregation of $cadN_H$ cells from untransfected S180 cells was inhibited by monoclonal antibodies to N-cadherin (not shown). Sorting-out was observed as early as 6 hr after centrifuging the cells onto the bottom of the plate (Fig. 1*A*, frame 2) and was apparent at different ratios of transfected vs. untransfected cells. Cells expressing lower amounts of a CAM (L_L or $cadN_L$ cells) segregated from untransfected cells to a lesser extent than cells expressing higher amounts (e.g., Fig. 1*A*, frame 5), but in all cases segregation was inhibited by Fab' fragments of anti-L-CAM antibodies and monoclonal antibodies to N-cadherin, respectively (Fig. 1*A*, frame 6).

Cells differing only in the level of expression of the same CAM (L_H/L_L and $cadN_H/cadN_L$; see Fig. 1*B*, frame 1) were also capable of sorting out. The degree of separation was similar to that for L_L/S180 and $cadN_L$/S180 pairings (Fig. 1*A*, frame 5), and the sorting-out was inhibited specifically by antibodies to L-CAM and N-cadherin (Fig. 1*B*, frame 2), respectively.

L_H cells segregated from $cadN_H$ cells (Fig. 1*B*, frame 3), and this process was inhibited by a combination of Fab' fragments to antibodies specific for L-CAM and monoclonal antibodies to N-cadherin (Fig. 1*B*, frame 4) but not by either antibody alone (Fig. 1*B*, frames 5 and 6). These results suggest that only one set of specifically transfected cells needs to interact via a cell adhesion system for sorting-out to occur, consistent with the segregation of transfected cells from the untransfected parent cell line.

Double transfectants (e.g., L_HcadN_L) segregated from untransfected cells (Fig. 1*C*, frame 1), and sorting-out was inhibited by a combination of Fab' fragments of anti-L-CAM antibodies and monoclonal anti-N-cadherin antibodies (Fig. 1*C*, frame 4); sorting-out was not inhibited when antibodies to the CAM expressed at low level (N-cadherin) were used (Fig. 1*C*, frame 3) and was only partially inhibited when antibodies to the CAM expressed at high level (L-CAM) were applied (Fig. 1*C*, frame 2). These observations indicated that each CAM by itself was sufficient to allow sorting-out.

Segregation between cell lines expressing both L-CAM and N-cadherin and cells expressing only L-CAM or N-cadherin depended on the levels of CAM expression. Segregation was observed when the expression of the unshared CAM was high in the doubly transfected cells, and the expression of the shared CAM was low in the singly transfected cells, as shown for example for $L_HcadN_L/cadN_L$ pairs (Fig. 1*C*, frame 5); sorting-out was also observed for L_HcadN_L/L_L, $L_HcadN_H/cadN_L$, $L_LcadN_H/cadN_L$, and L_LcadN_H/L_L combinations (not shown). In the $L_HcadN_L/cadN_L$ example shown (Fig. 1*C*, frames 5–8), sorting-out was inhibited by either Fab' fragments of polyclonal antibodies to the unshared L-CAM (Fig. 1*C*, frame 6) or a combination of these antibodies and monoclonal antibodies to the shared N-cadherin (Fig. 1*C*, frame 8). However, monoclonal antibodies to N-cadherin alone enhanced sorting-out (as indicated by the larger size of segregated cell clusters, Fig. 1*C*, frame 7) presumably because they blocked binding between the different cell lines. Unless antibody to the shared CAM was present, sorting-out was not observed when the level of expression was high for both CAMs in doubly transfected cells and for the CAM in singly transfected cells. For example, L_HcadN_H cells did not sort out from L_H (Fig. 1*C*, frame 9) unless Fab' fragments of polyclonal antibodies to the shared L-CAM were added (Fig. 1*C*, frame 10) to inhibit the binding of doubly transfected cells to singly transfected cells. In contrast, antibodies to both CAMs or monoclonal antibodies to the unshared N-cadherin did not allow sorting-out because the antibody mixture blocked all cell binding, and the anti-N-cadherin blocked the CAM activity that distinguished double from single transfectants (Fig. 1*C*, frames 11 and 12).

Table 3. Ability of cells expressing different levels of L-CAM and N-cadherin to sort out from each other

Cell line 2	Cell line 1						
	L_L	L_H	$cadN_L$	$cadN_H$	L_LcadN_H	L_HcadN_L	L_HcadN_H
S180	+	+	+	+	+	+	+
L_L		+	+	+	+	+	+
L_H			+	+	ND	ND	−
$cadN_L$				+	+	+	+
$cadN_H$					ND	ND	−
L_LcadN_H						ND	ND
L_HcadN_L							ND

ND, not determined; +, cell line 1 and cell line 2 had segregated when observed 16 hr after the start of a sorting-out assay; −, no segregation.

These results indicate that the capability of sorting-out specifically is conferred on transfected cells by both quantitative and qualitative differences in CAM expression. Most of these lines segregated when cocultured pairwise with each other: 21 of the 28 possible pairs were analyzed and sorting-out occurred in 19 of them (Table 3). Even cell lines that did not sort out (L_HcadN_H and L_H) were functionally distinguishable because L_H could be shown to segregate from $cadN_H$, while $cadN_HL_H$ did not.

Effects of Cytochalasin and Nocodazole on the Sorting-Out Process. To investigate the involvement of the cytoskeleton in CAM-mediated cell segregation (either by affecting CAM action or cell movement), sorting-out assays were performed in the presence of cytochalasin D to perturb microfilaments or nocodazole to disrupt microtubules. Sorting-out of L_H cells from S180 cells was completely inhibited by concentrations of 1 μg/ml to 0.3 μg/ml of cytochalasin D, and partial inhibition was observed at concentrations as low as 0.03 μg/ml. Sorting-out of L_H cells from S180 cells was only partially inhibited by nocodazole, as indicated by a decrease in the size of segregated cell clusters; a similar degree of inhibition was observed at concentrations ranging from 3 μg/ml to 0.1 μg/ml. Controls indicated that cytochalasin and nocodazole caused no irreversible toxic effects at the concentrations used.

DISCUSSION

To begin to define the mechanisms by which a small number of CAMs can help form various discrete cell populations, we

examined the process of cell sorting-out *in vitro* by analyzing the behavior of cell lines transfected with cDNAs coding for two similar calcium-dependent CAMs, L-CAM and N-cadherin (A-CAM). These CAMs, while closely related in structure (10, 19), did not appear to bind to each other. When cells expressing one of these molecules were mixed with untransfected cells or with cells expressing the other CAM, they sorted into discrete populations. The process was dependent on CAM binding activity; the presence of either CAM was sufficient for cell sorting. Segregation also occurred between cells expressing different levels of the same CAM or different combinations of CAMs, consistent with the notion (1, 2, 14) that the activity of a few CAMs can help generate a rather large variety of cell patterns.

Significant levels of CAM expression did not always lead to cell segregation—for example, in the pairing $L_H cadN_H/L_H$. The most likely explanation of these results is that, while sorting-out was directly related to the amount of CAM expressed at the cell surface, L-CAM-mediated binding so dominates the cellular interactions in this case that adding N-cadherin has little additional effect.

The data presented here and earlier results clearly demonstrate that CAMs can influence the ability of cells to sort into different populations. However, sorting-out is a complex process in which both cell adhesion and cell movement play central roles, and therefore other structural elements, including cytoskeletal components and cell junctions, are likely to be involved. Consistent with this view, both nocodazole and cytochalasin D inhibited cell sorting-out. Cytochalasin D disrupts microfilaments, and earlier studies showed that L-CAM, A-CAM, and N-cadherin colocalize with cortical actin, that L-CAM activity is dependent on its interaction with cytoplasmic elements (13, 20–23), and that adhesion among S180L and S180cadN cells is inhibited directly by cytochalasin D (F. Matsuzaki, R.-M.M. and G.M.E., unpublished data). Therefore, cytochalasin D probably affects both cell adhesion and cell movement. In contrast, nocodazole, which disrupts microtubules, probably acts primarily by influencing cell movement; its effect was weaker than that of cytochalasin D, and it did not appear to influence cell adhesion directly.

The demonstration that factors other than differences in CAM specificity can regulate sorting-out of cells indicates that, *in vivo*, differential expression of both the level and the type of a small number of CAMs can lead to a larger number of different cell collectives. The contribution of each variable clearly depends on cellular driving forces (movement, shape, charge, etc.), on the modulation of CAM activity at the cell surface (24) and on the nonlinear properties (25) of CAM binding. A more detailed investigation will be necessary to establish the minimal differences in the levels of CAM expression that will lead to segregation; this parameter is likely to depend on both the assay and the phenotypic properties of the cells used.

While differences in CAM specificity are undoubtedly essential in the formation of tissue boundaries during development (1), the results presented here indicate that the amounts and combinations of CAMs expressed can also be strong factors in pattern formation. These observations support the idea that *in vivo* differences in the levels of expression of a few CAMs may lead to a rather large set of patterns of functionally distinct cell types within tissues.

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